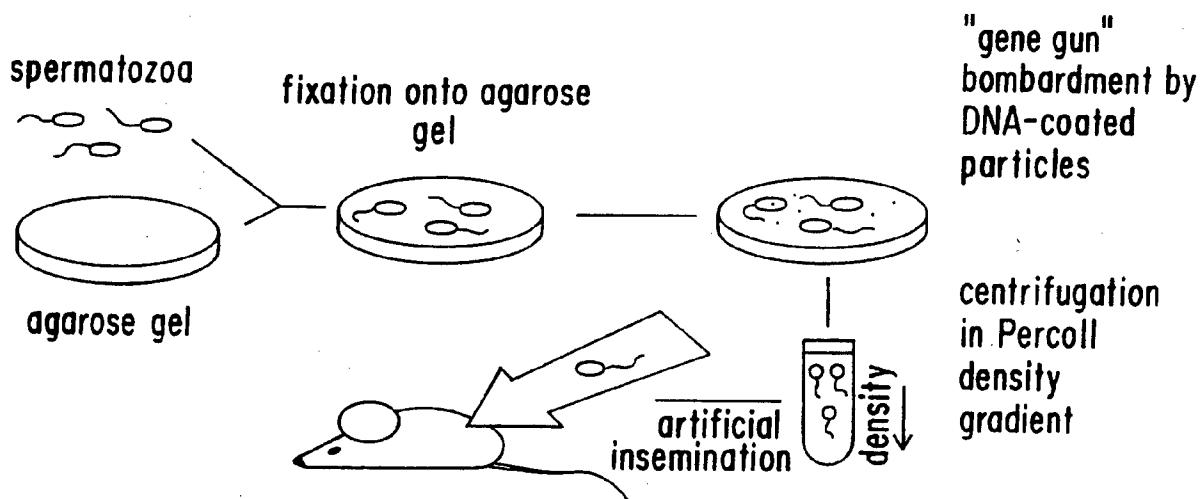




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :  C12N 15/00, 15/87, 15/23 A01K 67/027, C12N 5/10		A1	(11) International Publication Number: WO 93/24626  (43) International Publication Date: 9 December 1993 (09.12.93)
(21) International Application Number: PCT/US92/04426  (22) International Filing Date: 28 May 1992 (28.05.92)		(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).	
(71) Applicant (for all designated States except US): SCIENTIFIC DIMENSIONS USA, INC. [US/US]; 185 Alewife Brook Parkway, Cambridge, MA 02138 (US).		Published With international search report.	
(72) Inventors; and (75) Inventors/Applicants (for US only) : LARIONOV, Oleg [RU/RU]; DOBROVOLSKY, Vasily N. [RU/RU]; Shemyakin Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya, 16/10, GSP Moscow V-437, 117871 (RU).			
(74) Agents: GREASON, Edward, W. et al.; Kenyon & Kenyon, One Broadway, New York, NY 10004 (US).			

(54) Title: TRANSGENIC ANIMAL PRODUCTION WITH BIOLISTICALLY TRANSFORMED SPERMATOZOA



## (57) Abstract

A method of producing transgenic animals which comprises binding DNA which expresses a desired trait to a particle, inserting the carrier particle with bound DNA into spermatozoa, and fertilizing a sufficiently related egg. Also disclosed is a method for reversibly fixing spermatozoa on a slightly dried agarose gel so that they can be bombarded by DNA-laden particles from a gene gun.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	L.I.	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

## TRANSGENIC ANIMAL PRODUCTION WITH BIOLISTICALLY TRANSFORMED SPERMATOZOA

### FIELD OF THE INVENTION

The present invention relates to methods of creating transgenic animals which contain cloned genes taken up into spermatozoa such that the spermatozoa function as vectors when fertilizing an egg of a sufficiently related species. The spermatozoa take up the cloned DNA, for example, as a result of being bombarded by DNA-laden particles from a gene gun while fixed to an agarose gel. The cloned sequence is transmittable to offspring.

### BACKGROUND OF THE INVENTION

Introducing foreign DNA into somatic and germinal cells of animals is well established [Palmeter & Brinster, *Ann. Rev. Genet.* 20, 465-499 (1986)]. The most widely used method is the direct microinjection of foreign DNA into pronuclei of fertilized eggs [Gordon et al., *Pros. Natl. Acad. Sci. USA* 77, 7380-84 (1980)]. However, receiving zygotes at the developmental (two pronuclei) stage suitable for microinjection is problematic. Introducing foreign DNA into mammalian genome may also be done using transfected embryo-derived stem (ES) cells, [Lovell-Badge et al., *Cold Spring Harb. Symp. Quant. Biol.* 50, 707-11 (1985)], although obtaining live-stock ES cell lines is extremely difficult. The insertion of genes into the germ line via retroviral vectors has also been improved [van der Putten, *Pros. Natl. Acad. Sci. USA* 82, 6148-52 (1985)]. Nonetheless, because of the structural characteristics of retroviruses, using them to construct vectors presents grave difficulties.

Only recently has cloned DNA been introduced directly into spermatozoa [Lavitrano et al., *Cell* 57, 717-723 (1989)]. Indeed, mature

mouse sperm cells incubated in an isotonic buffer with cloned DNA will capture DNA molecules. Thus, sperm cells may be used as vectors for introducing foreign DNA into eggs at fertilization. Mouse eggs can be fertilized *in vitro* by sperm incubated in the cloned DNA solution then 5 transferred, at the two-cell stage, to the oviducts of foster mothers.

Approximately 30% of 250 screened mice revealed clear sequence homology to the cloned DNA sequence. The key to this method is the capture of foreign DNA by spermatozoa through exposure in an isotonic solution.

Foreign genes have also been introduced into spermatozoa using 10 liposomes, [Hofer, *Europ. Biotech. Newsletter* 47, 4704 (1988)]. The spermatozoa loaded by the liposome method have been used to transform egg or somatic cells under intra- or extracorporeal conditions to produce hepatitis surface antigen and a rat immunoglobulin. [German Pat. No. WO 87/05325].

15 Transgenic animals also have been produced by introducing foreign DNA into some tissues of adult animals or fetuses by means of: a) a retrovirus vector carrying the DNA of interest; b) CaPO<sub>4</sub> mixed with the DNA of interest, or c) electroporation, [U.K. Patent No. 2,223,755]. Unfortunately, use of these treatments results in animals which do not 20 transmit foreign DNA to their progeny. Finally, a transgenic non-human mammal has been produced where germ cells and somatic cells contain a recombinant activated oncogene sequence introduced into said mammal at an embryonic stage, [U.S.P. 4,736,866 (The "Harvard Transgenic Mouse")].

"Gene guns" have been manufactured to facilitate introduction of 25 cloned DNA into various types of cells by bombarding them. One typical example of a gene gun, Dupont's PDS-2000, utilizes microparticles (usually made of tungsten or gold) laden with the cloned DNA to pierce a cell membrane and introduce the DNA into the cell. Specifically, Dupont's PDS-2000 has been used on muscle cells, human cultured cells, fibroblasts, chinese 30 hamster ovaries and lymphocytes to cause the up-take of cloned DNA. However, the gene gun requires its target cells to be exposed on a support

surface to prevent particle breaking in the medium and for efficient DNA placement. The initial conditions for using Dupont's PDS-2000 vary, but Dupont suggests the following conditions to optimize bombardment: cells should be maintained at 15 inches of Hg, for as brief a period as possible (1-5 min.) optimizing vacuum pressure up to 27 inches of Hg in three such integrals. This increases the speed of the microcarriers. If cells become damaged or cell survival decreases, vacuum levels should be reduced. In addition, as the distance between the accelerator and the stopping plate increases, the velocity of the microcarrier decreases, and dispersion increases.

10 Thus, different ranges of distance should be tried for comparison. The microcarrier must be placed optimally in the acceleration tube so the velocity is optimized. Microcarriers should be one-tenth the diameter of the cell. M5 (average is 0.35  $\mu\text{m}$ ) and M10 (average is 0.73  $\mu\text{m}$ ) tungsten and the one  $\mu\text{m}$  gold particles have all been used successfully on animal cells in tissue culture

15 [DuPont Biolistic™ PDS-1000 Instruction Manual]. The successful bombardment of live sperm has not been yet reported.

#### DESCRIPTION OF THE FIGURES

Figure 1 is a schematic diagram of a recombinant vector used to transfer sperm in a preferred embodiment of the present invention.

20 Figure 2 summarizes the various general steps of certain preferred embodiments of the invention.

Figure 3 is a sketch of the vector construction used on the BLG gene for human gamma interferon expressing in mammary gland of transgenic animals.

25

#### SUMMARY OF THE INVENTION

The purpose of this invention is to create a universal, simple, efficient and inexpensive method for production of transgenic live stock transmitting foreign genes to their progeny. This method will obviate the difficulties

inherent with recovering early embryos and treating donor and acceptor animals.

In accordance with the present invention, a method of producing a transgenic animal is disclosed where DNA which expresses a desired trait is 5 bound to a carrier particle, the carrier particle is inserted into spermatozoa, and the spermatozoa is used to fertilize an unfertilized egg from a species sufficiently related to the one from which said spermatozoa was collected. Species from which spermatozoa and an unfertilized egg are collected are "sufficiently related" if fertilization of the egg by the sperm can result in a 10 viable embryo. In certain preferred embodiments, a gene gun can be used to insert a carrier particle bound with DNA into spermatozoa. Any method of inserting the DNA-laden carrier particle can be used, although bombardment with a gene gun is preferred.

The carrier particles to which the cloned DNA is bound are tungsten 15 or gold. Any other material may be used to bind the cloned DNA if it will sufficiently survive the chosen insertion method and properly present the DNA for integration into the spermatozoan genome.

Fertilization of the unfertilized egg by the spermatozoa containing the inserted DNA can be by *in vitro* fertilization, by artificial insemination (for 20 example, in the oviduct), or by any other means for uniting genetic material from gametes.

A method is also disclosed for reversibly fixing sperm so that they may be successfully bombarded by DNA-laden particles. Sperm recovered from epididymis is dissolved at room temperature in a small volume of medium, 25 then dropped onto the surface of slightly dried 1% agarose gel. After liquid evaporation, the spermatozoa become fixed on the gel. The bombardment process can occur for three to five minutes, after which a medium is added to the gel so the spermatozoa can regain motility.

DETAILED DESCRIPTION OF THE INVENTION

This invention, in certain preferred embodiments, consists of the following steps (see fig. 2): 1) fixing spermatozoa onto agarose gel, 2) bombarding fixed spermatozoa (head dimensions are approximately 2x5 $\mu$ m)

- 5 by DNA-coated particles using a gene gun (gold or tungsten particles with diameter of 0.2-0.5 $\mu$ m); 3) harvesting bombarded spermatozoa and using centrifugation in a Percoll density gradient to isolate spermatozoa containing DNA-coated particles; and 4) using isolated spermatozoa for artificial insemination. After fertilizing an egg with spermatozoa carrying DNA-coated
- 10 particles, a male pronucleus will form, followed by dissociation of the foreign DNA from the particle surface and integration of it into the genome. The particle will be torn away during embryo cleavage.

A vector was constructed by amplifying [using polymerase chain reaction (PCR)] of the 3'-end of the beta-lactoglobulin (BLG) gene

- 15 comprising the last intron and poly(A) site and inserting of that fragment into a Pvu II site (fig. 1). More specifically, the 11.3kb DNA fragment comprising the 4.9kb transcription unit, the 4.8kb 5' flanking sequence and the 1.6kb 3' flanking sequence of the BLG gene was cloned (using standard methods of genetic engineering [Davis et al., Basic methods in Molecular Biology].
- 20 Elsivier, N.Y. (1986)] into plasmid pBRUC318 (fig. 3.A) from a sheep genomic library (in phage lambda-EMBL3) using as probes two partially overlapping synthetic oligodeoxyribonucleotides Seq. Id. No:1 and Seq. Id. No:2 complementary to the coding sequence of the BLG gene first exon [Ali S., Clark, A.J., J. Mol. Biol. 199, 415-426 (1988)]. The PvuII site resides
- 25 within the first exon (in untranslated region). Human gamma-interferon cDNA was inserted into the SnaBI site.

A vector was created for human gamma-interferon (g-IFN) expression in the mammary gland. A fragment of the 3' end of the BLG gene, comprising the last intron and poly(A) site, was amplified using polymerase

- 30 chain reaction (PCR) (fig.3.B.). For this purpose two synthetic oligonucleotides Seq. Id. No:3 and Seq. Id. No:4 were used as primers. They

contained recognition sequences for restriction endonucleases SnaBI and Hind III at their 5' ends and were complementary to flanks of this fragment. PCR conditions were: 100pmol of each primer, 0.1ng of target DNA (BLG gene), 67mM TrisHCl pH8.8, 16.6mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 6.7mM MgCl<sub>2</sub>, 10mM 5 DTT, 0.2mM of each dNTP, 100μg/ml BSA, Taq I DNA polymerase 2un, H<sub>2</sub>O to final volume of 100μl. A total of 30 cycles were performed, each cycle being 30 sec. at 95°C, 30 sec. at 65°C, 2 min. at 72°C. An amplified fragment (616bp) bearing newly created SnaBI (at its 5' end) and Hind III (at its 3' end) sites was ligated into the Pvu II site that resides within the 10 untranslated region of the BLG gene first exon using standard methods of molecular cloning (fig.3.C). g-IFN cDNA [received from Prof. E.D. Sverdlov (Institute of Bioorganic Chemistry, USSR Academy of Sciences)] that contained initiating and terminating codons was inserted into the SnaBI site (fig.3.D). This DNA construct is used to load carrier particles.

15 DNA is bound to tungsten or gold particles using the following procedure: 1.25 mg of tungsten is heated in 95% EtOH at 65°C for four hours. 25 μg of DNA, 2.5M CaCl<sub>2</sub> and 0.1M spermidine are combined to a final volume of 575μl (final concentration of 1.1M CaCl<sub>2</sub> and 8.7mM spermidine). The mixture is vortexed at 4°C for 10 minutes, then centrifuged 20 at a low speed (500 rpm) for 5 minutes. Approximately 550μl of supernatant is then removed and the remaining 25 μl is separated into 1μl aliquots for loading onto a macrocarrier. It is better to use gold particles as tungsten may be toxic. DNA is bound to gold particles according to the method described by Du Pont. [Newsletter, *A Publication of the Du Pont Company*, 1, 1-4 25 (1990)].

To create transgenic animals, spermatozoa are bombarded by accelerated DNA-coated microparticles from a gene gun, such as the PDS-2000. For the purpose of bombardment, spermatozoa are reversibly fixated on a low melting point agarose gel. To accomplish the reversible fixation, 30 sperm is collected from the epididymis, and dissolved at room temperature in a small volume (approx. 50-150μl) of M2 medium [Quinn, et al., *J. Reprod.*

*Fertil.* 66, 161-168 (1982). The sperm solution is then dropped onto the surface of a slightly dried 1% agarose gel (Sea Plaque agarose, FMC, USA).

After liquid evaporation, the spermatozoa are fixed on the gel. Three to five minutes later, during which time bombardment with foreign DNA can occur,

- 5 M2 medium (50-250 $\mu$ l) is added to the gel to reverse fixation. About 60-80% of fixed spermatozoa regain motility in the liquid phase.

Spermatozoa containing DNA-coated particles are separated from other spermatozoa by density gradient centrifugation. The density gradient is formed in 10-15ml centrifuge tubes during high-speed centrifugation (15 min.

- 10 29000g) of Duldecco's PBS containing 60-90% of Percoll. Bombarded spermatozoa washed over the surface of the gel are layered onto preformed density gradient tubes and after low-speed centrifugation (20 min. 400g) at room temperature, they are separated on fractions depending on their floating density. Spermatozoa which contain particles are higher density so
- 15 they concentrate closer to the bottom of the tube. The desired spermatozoa are then collected by puncturing of the tube bottom.

The DNA-laden spermatozoa are then used to artificially inseminate a female specimen or to fertilize eggs by other known means. For example, with mice the preferred method of fertilization is to introduce DNA-laden

- 20 spermatozoa directly into the oviduct of pseudopregnant females (females mated by sterile males) or transfer the selected spermatozoa (from density gradient tube) to the oviduct or the uterus using fine glass pipet (outer diameter 100-120 $\mu$ m) This procedure is analogous to the procedure of embryo transfer into the uterus [Hogan et al., *Manipulating the Mouse Embryo*. (Cold Spring Harbor Laboratory, N.Y., 142-145 (1986))]. The normal artificial insemination of a mouse female is difficult due to particularities of organization of mouse female reproductive organs. In vitro fertilization is also possible.

For livestock routine artificial insemination is preferred, using the

- 30 same gene gun technology applied with mouse spermatozoa. In vitro fertilization is also possible.

Integration of foreign DNA into the murine (or other) genome is analyzed using PCR. Two oligonucleotides (e.g., Seq. Id. No:5 and Seq. Id. No:6) are used as primers for checking the integration of the g-IFN cDNA. They are complementary to g-IFN cDNA and form 252bp DNA fragments

5 during PCR. PCR conditions: 20pmol of each primer, 0.1-0.5 $\mu$ g of mouse genomic DNA, 0.2mM of each primer, 50mM KCl, 10mM TrisHCl pH8.14, 1.5mM MgCl<sub>2</sub>, 100 $\mu$ g/ml gelatin, Taq I DNA polymerase 1-1.5 units, and H<sub>2</sub>O to final volume of 20 $\mu$ l. The mixture runs for 30 cycles, each being: 30sec 94°C, 30sec 55°C, 30sec 72°C. 10-15 $\mu$ l aliquots of reaction mix is

10 electrophoresed on 1.5% agarose gel in the presence of ethidium bromide at 100V for 30min and viewed under UV light.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Larionov, Oleg  
Dobrovolsky, Vasily N.(ii) TITLE OF INVENTION: A NEW METHOD FOR TRANSGENIC ANIMAL  
PRODUCTION

(iii) NUMBER OF SEQUENCES: 6

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Paul Lempel, Esq.  
(B) STREET: One Broadway  
(C) CITY: New York  
(D) STATE: New York  
(E) COUNTRY: USA  
(F) ZIP: 10004

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US N/A  
(B) FILING DATE: 16-SEP-1991  
(C) CLASSIFICATION:

## (vii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lempel Esq., Paul  
(B) REGISTRATION NUMBER: 21,198  
(C) REFERENCE/DOCKET NUMBER: 1036/61

## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (212) 425-7200

10

(B) TELEFAX: (212) 425-5288

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCGGGTCCA GGCCATCATC GTC

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTCATGGTC TGGGTGACGA TGATGG

23

26

## (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TACGTAGGTG AGCCCTGGCC GGTGCG

## (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAGCTTCCAG CAAAGACTCA GAAGG

## (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

12

25

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AATGCAGGTC ATTCAAGATGT AGCGG

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGAATAATTAA GTCAGGCTTT CGAAG

25

CLAIMS

We claim:

- 1        1.     A method of producing a transgenic animal said method  
2 comprising:
  - 3            A.     binding DNA which expresses a desired trait to a carrier  
4            particle;
  - 5            B.     inserting said carrier particle with said DNA into  
6            spermatozoa of a selected species; and
  - 7            C.     fertilizing an unfertilized egg from a species sufficiently  
8            related to said selected species.
- 1        2.     The method of claim 1 wherein a gene gun is used to insert  
2     said carrier particle bound with DNA into said spermatozoa.
- 1        3.     The method of claim 2 wherein said carrier particle is made of  
2     a material selected from the group consisting of tungsten and gold.
- 1        4.     The method of claim 1 wherein said unfertilized egg is fertilized  
2     *in vitro*.
- 1        5.     The method of claim 1 wherein said unfertilized egg is fertilized  
2     by artificial insemination.
- 1        6.     The method of claim 1 further comprising separating  
2     spermatozoa containing said carrier particle from spermatozoa not carrying  
3     said carrier particle.
- 1        7.     The method of claim 6 wherein said spermatozoa are separated  
2     by centrifugation.

1           8.    The method of claim 2 further comprising, prior to inserting  
2    said carrier particle, reversibly fixing said spermatozoa to a support.

1           9.    The method of claim 8 wherein said spermatozoa are fixed by a  
2    process according to claim 10.

1           10.   A method for reversibly fixing spermatozoa said method  
2    comprising:  
3               (a)   diluting a sample of spermatozoa in a first medium;  
4               (b)   contacting said sample with an agarose gel; and  
5               (c)   evaporating the liquid portion of said first medium such  
6    that said spermatozoa become fixed to said gel.

1           11.   The method of claim 10 further comprising reversing the  
2    fixation of said sperm by adding a second liquid medium to said spermatozoa.

1           12.   The method of claim 10 which the first medium is a M2  
2    medium.

1           13.   The method of claim 10 wherein the second medium is an M2  
2    medium.

1           14.   The method of claim 10 wherein said gel is 1% agarose gel.

1           15.   A method for reversibly fixing spermatozoa comprising:  
2    collecting sperm from the epididymis and dissolving it at room temperature in  
3    a small volume (approx. 50-150 $\mu$ l) of M2 medium; dropping the sperm  
4    solution onto the surface of a slightly dried 1% agarose gel; allowing time for  
5    liquid evaporation so that the spermatozoa become fixed on the gel;  
6    bombarding the fixed spermatozoa for three to five minutes; and adding M2  
7    medium (50-250 $\mu$ l) to the gel to reverse fixation.

1           16. The method of claim 15 wherein reversibly fixed spermatozoa  
2 are fixed according to the method of claim 10.

1           17. A DNA-laden spermatozoon made according to the method of  
2 claim 18.

1           18. A method of production of DNA-laden spermatozoa  
2 comprising:

3                   (a) the method for reversibly fixing spermatozoa according  
4 to the method of claim 10; and

5                   (b) the method for reversing the fixation according to the  
6 method of claim 11.

1/3

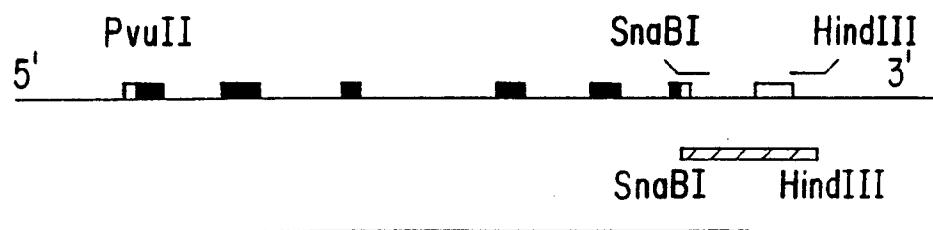


FIG. 1

**SUBSTITUTE SHEET**

2/3

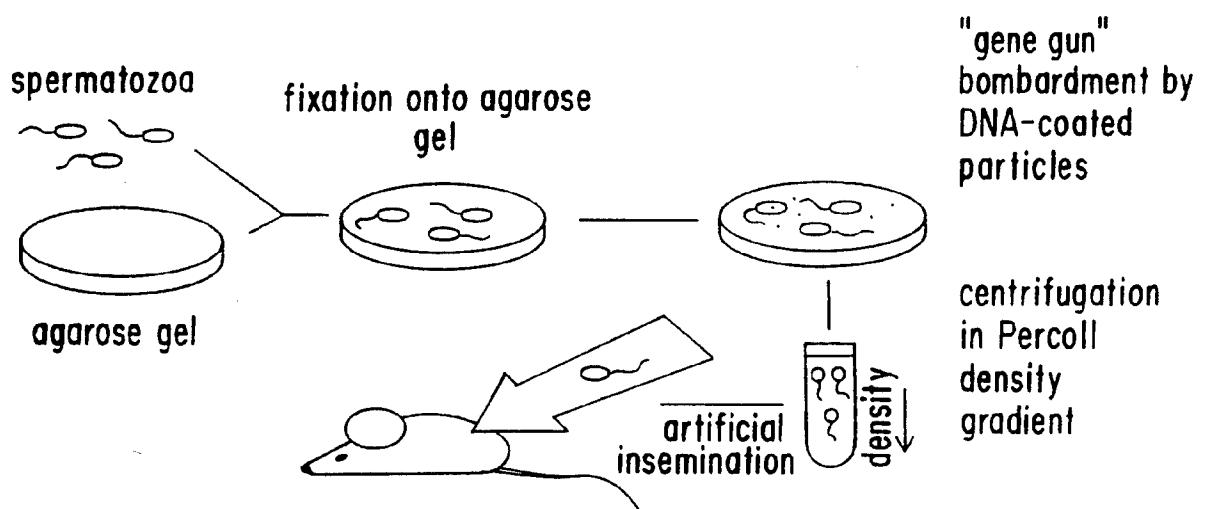


FIG. 2

SUBSTITUTE SHEET

3/3

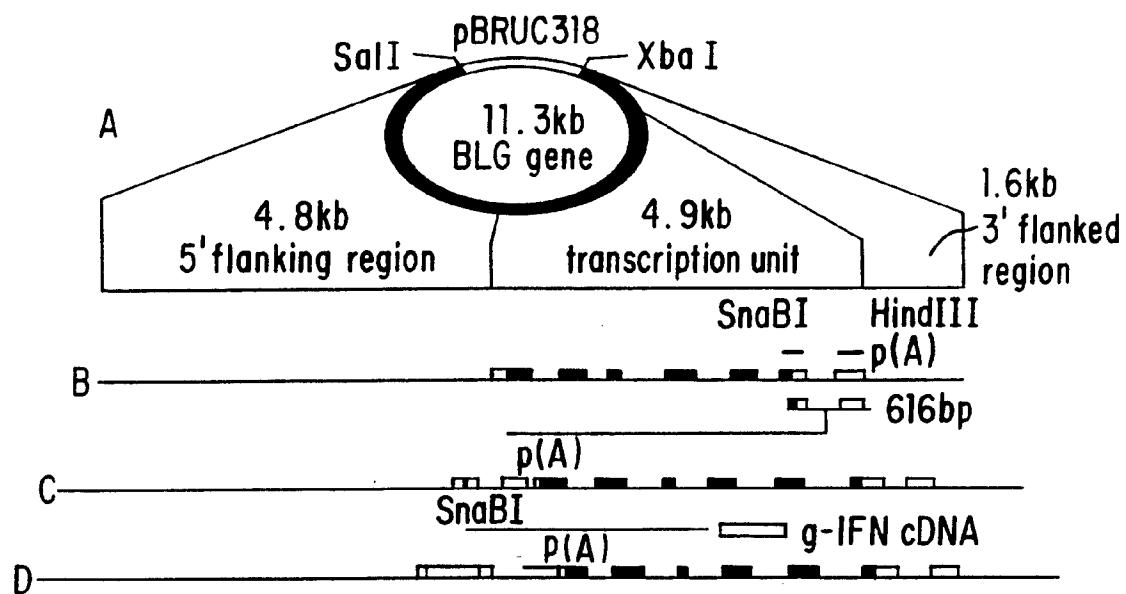


FIG. 3

SUBSTITUTE SHEET

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/04426

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/00; C12N15/87; C12N15/23; A01K67/027  
C12N5/10

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO,A,9 119 781 (AGRACETUS) 26 December 1991 see page 6, line 25 - page 12, line 30 ----	1-3
X	WO,A,8 705 325 (TRANSGENE) 11 September 1987 see the whole document ----	1
Y	EP,A,0 431 839 (SCOPUS-GENETICS) 12 June 1991 see the whole document ----	1-5
Y	WO,A,9 008 192 (CONSIGLIO NAZIONALE DELLE RICERCHE) 26 July 1990 see the whole document ----	1-5
Y	WO,A,9 118 991 (E. I. DUPONT DE NEMOURS) 12 December 1991 ----	1-3
		-/-

<sup>6</sup> Special categories of cited documents :<sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

<sup>7</sup> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<sup>8</sup> "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step<sup>9</sup> "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<sup>10</sup> "A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search 20 JANUARY 1993	Date of Mailing of this International Search Report 12.02.93
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer CHAMBONNET F.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category <sup>a</sup>	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,9 107 487 (DUKE UNIVERSITY) 30 May 1991 see the whole document ----	1-5
X	WO,A,9 100 359 (AGRACETUS) 10 January 1991 see page 5, line 10 see the whole document ----	1-3
Y	WO,A,9 003 439 (AMGEN) 5 April 1990 see the whole document ----	1-5
X	WO,A,9 008 832 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 9 August 1990 see the whole document -----	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

US 9204426  
SA 60799

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 20/01/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9119781	26-12-91	US-A-	5149655	22-09-92
		AU-A-	8210891	07-01-92
		CA-A-	2065361	22-12-91
		EP-A-	0487711	03-06-92
-----				
WO-A-8705325	11-09-87	DE-A-	3636991	24-09-87
		AU-B-	597965	14-06-90
		AU-A-	7167987	28-09-87
		EP-A-	0258427	09-03-88
		JP-T-	63502638	06-10-88
		ZA-A-	8701468	17-08-87
-----				
EP-A-0431839	12-06-91	AU-A-	6767290	04-07-91
		CA-A-	2031344	04-06-91
		JP-A-	3266983	27-11-91
-----				
WO-A-9008192	26-07-90	AU-A-	4810790	13-08-90
		EP-A-	0453458	30-10-91
		JP-T-	4504352	06-08-92
-----				
WO-A-9118991	12-12-91	AU-A-	7878891	31-12-91
-----				
WO-A-9107487	30-05-91	AU-A-	6964191	13-06-91
		EP-A-	0500799	02-09-92
-----				
WO-A-9100359	10-01-91	AU-A-	5856790	17-01-91
		CA-A-	2019676	26-12-90
		EP-A-	0431135	12-06-91
		JP-T-	4500314	23-01-92
-----				
WO-A-9003439	05-04-90	US-A-	5162215	10-11-92
		AU-A-	4312389	18-04-90
		EP-A-	0393176	24-10-90
		JP-T-	3503485	08-08-91
-----				
WO-A-9008832	09-08-90	AU-A-	5027690	24-08-90
		EP-A-	0455695	13-11-91
		GB-A,B	2228487	29-08-90
		JP-T-	4503004	04-06-92
-----				